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TITLE: Population Based Assessment of MHC Class I Antigens Down Regulation as Markers of Increased Risk for Development and Progression of Breast Cancer From Benign Breast Lesions

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13. Abstract ***(Maximum 200 Words)*** *(abstract should contain no proprietary or confidential information)*

Despite advances in chemotherapy and radiation therapies, advanced breast cancer still carries a high mortality rate. The need for effective therapies is urgent. The overall aim of this research proposal is to recognize early markers of disease and their interaction with other epidemiological risk factors that can serve as risk indicators for subsequent development of breast cancer from precancerous lesions, and as prognostic markers for progression from primary to metastatic disease. The major histocompatibility complex (MHC) class I molecules are found on the cell membrane of all cells in the body and are involved in intercellular communications and in complex interactions with the immune system. Cancer cells with reduced or aberrant MHC molecules have been shown to evade immune surveillance and become selected for cancer progression and spread of disease to distant sites of the body. About half of all breast cancers have complete loss or reduced level of MHC class I molecules and this finding has been associated with increased tumor invasiveness and more aggressive cancers with poorer outcome. The outlined studies are expected to better define the clinical significance of abnormal MHC class I molecules in precancerous and invasive breast lesions as markers of immunological events that could affect survival, selection, and outgrowth of precancerous cells, and their subsequent progression to breast cancer. These MHC losses could also mark more aggressive tumors and thus contribute to selection of appropriate treatments in individual cases.

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major histocompatibility complex (MHC) class I molecules, disease progression markers, survival, breast cancer

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INTRODUCTION

It has been known for some time that malignant transformation of cells is frequently associated with abnormalities in the expression of MHC class I antigens (1). These abnormalities appear to play a role in the clinical course of the disease (1) and to have a negative effect on the outcome of T cell-based immunotherapy for malignant diseases (2, 3). In breast lesions examined for expression of MHC class I, approximately half (51%) of carcinomas had an abnormally low content of HLA-A, -B, and -C determinants (4). Down regulation of HLA class I antigens in breast carcinomas may be more frequent than previously reported suggesting that alterations of HLA class I could represent an important step associated with tumor invasion providing tumor cells with the ability to escape recognition by T-lymphocytes (5). The overall aim of our research is to better define the role of MHC class I antigen loss and its interaction with other histo-pathologic and epidemiological factors that can serve as risk indicators in the progression of primary breast cancer to metastatic disease.

BODY: Statement of Work

TASK 1:

In women with primary and metastatic lesions of the breast to determine whether HLA Class I antigen loss and down regulation is greater in those with late stage and metastatic disease than in women with early stage disease (months 1-48); to determine whether among women with concurrent preneoplastic lesions and breast tumors HLA Class I antigen loss or down regulation is more frequent in the tumor than in the pre-neoplastic lesion (1-54); association with histopathologic characteristics of the lesions, including estrogen and progesterone receptor status (months 1-54); and disease survival (1-58).

a: Begin construction of the breast cancer cohort (3000 cases). The Pathologist Dr.Raju and the P.I will begin screening breast cancer cases for delineation into Stage1-IV, and for the presence of concurrent lesions of benign proliferative and cancer lesions, together with normal breast tissue. We will design appropriate forms to record histopatholgical and clinical data based on our current NIH project forms and instruments (Instruments section in original grant)

b: Retrieval of H & E slides for cases

c: Review of slides

d: selection of tumor blocks and sectioning of tissue for immunohistochemistry assays

e: begin HLA class I immunoassays, as slides become available

f: Continue construction of the breast cancer cohort, the concurrent lesion cohort and the histopathologic data gathering. See Pathology Review Form (PRF) (Instruments Section) for histopathologic parameters.

g: continue HLA class I immunoassays as additional cases are entered into the cohort

h: Annual reports will be written

i: Initial manuscripts on the PBD cohort will be written

PROGRESS (January 1, 2002- December 31, 2002):

1. The finalized Pathology Review Form (PRF) has been converted into the Teleform version for electronic data entry Appendix A)
2. Thus far over 3000 pathology reports have been obtained
3. H& E slides have been retrieved from the pathology archives for an additional 700 cases, for a total of 950 cases.
4. 525 additional cases have been reviewed by Dr. Raju on PRF forms for a total of 750 cases
5. Selection of tumor blocks completed for an additional 410 cases bringing the total cases to 580 thus far.
6. Sectioning of tissue for immunohistochemistry assays: completed for 330 cases.
7. HLA class I immunoassays: completed for 196 cases
8. Medical abstraction form: completed in 310 cases
9. Presented two papers at the 13th International Histocompatibility Workshop, Victoria, BC May 11-May 16, 2002

TASK 2
Final analysis and report writing (months 56-60)

a: Final analysis of epidemiological risk factor data, histopathological and clinical data and HLA expression results will be performed.

b: A final report and additional manuscripts on the breast cancer cohort will be prepared

Progress: PENDING

KEY RESEARCH ACCOMPLISHMENTS

- A major accomplishment has been the verification and validation of our constructed breast cancer cohort. The Henry Ford Health System Tumor Registry data, which is the source of our study cohort starting from 1981 through 2000 was validated and verified for vital patient information against the SEER data. We have so far acquired a total patient database of 4,900 validated and verified breast cancer cases.
- Presented two papers at the 13th International Histocompatibility Workshop, Victoria, BC, May 11-May 17, 2002
  - A: A multicenter validation of immuno assays with HLA antibodies
  - B: Working with archival tissues: Interpretation of Gene expression of MHC class 1 and II and Loss of Heterozygosity at the HLA locus at 6p21.

- Completed a total of 1,500 slide (130 cases) for HLA downregulation

- Acquisition of a Tissue Array system through departmental funds to accommodate a much higher throughput for immunostained tissues.

REPORTABLE OUTCOMES

1. ABSTRACTS/PRESENTATIONS/MANUSCRIPTS

a: HLA CLASS I AND II ANTIGEN EXPRESSION IN BREAST CARCINOMAS. M. Worsham, U Raju, S Ferrone. Department of Pathology, Henry Ford Health System, Detroit, Department of Immunology, Roswell Park Cancer Institute, Buffalo, 13th International Histocompatibility Conference, Victoria, BC, May 11-May 17, 2002

b: HLA ANTIGEN EXPRESSION IN BREAST CANCER: A MULTICENTRIC STUDY UTILIZING FORMALIN-FIXED PARAFFINIZED TISSUES. M J. Worsham[1], R. Nanavati[1], U. Raju[1], S.R. Wolman[2], T. Cabrera [3], F. Garrido [3], E. A. Repasky[4], B. Hylander[4], M. Feenstra [5], M.Verdaasdonk[5], M.Schipper[5], M.Tilanus[5], S.Ferrone[4]. [1] Cancer Genetics Research, Department of Pathology, Henry Ford Health Systems, Detroit, MI, 48202, USA [2] Uniformed Services Univ., of the Health Sciences, Bethesda, MD 20814, USA, Hosp., Univ., Virgen de las Nieves, Granada, Spain, [4] Roswell Park Cancer Institute, Buffalo, NY 14263, [5] Univ., Hosp., Utrecht, The Netherlands.
Manuscript in final revision for submission to "Tissue Antigens" (Appendix B)

c: SELECTIVE DOWNREGULATION OF HLA CLASS I BW4 NEGATIVE ANTIGENS IN HUMAN LEUKEMIA SUGGEST A COMBINED ESCAPE FROM T- AND NK CELL ATTACK. Demanet C[1], Mulder A[2], Deneys V[3], Worsham MJ[4], Claas F [2], Ferrone S [5]. [1]HLA laboratory, Academic Hospital VUB, Brussels, Belgium, [2]Immunohematology, University Medical Center, Leiden, the Netherlands, [3]Immunohematology, UCL, Brussels, Belgium, [4]Dept. of Otolaryngology, Henry Ford Hospital, Detroit, USA, [5]Dept. of Immunology, Roswell Park Cancer Institute, Buffalo, New York, USA
Manuscript in final revision for submission to "Blood" (Appendix B)

CONCLUSIONS:

HLA CLASS I AND II ANTIGEN EXPRESSION IN BREAST CARCINOMAS

In the present study we have compared the expression of HLA antigens in breast carcinoma epithelial cells and in autologous normal epithelial cells utilizing 44 formalin-fixed, paraffin-embedded lesions. An initial cohort of 44 breast cancer

cases indicated an altered expression of class I HC10 in 40/44 cases: 27 showed down regulation (decreased expression or complete loss), 8 upregulation (overexpression), 5 heterogeneity for loss, and 4 indicated no change from normal expression. Loss of B2-microglobulin expression (L368) was observed in 18/33 cases. Concordance for altered expression of HC10 and L368 was noted in 23 cases, the most frequent being loss (14/23).

A relationship was found between HLA class I antigen expression and the degree of differentiation of malignant cells. Generally, neither breast carcinoma cells nor normal mammary epithelial cells were stained by anti-HLA class II mAb LGII-612.14. Lymphocytes and dendritic cells were the consistently stained by mAb LGII-612.14 in the tissue sections analyzed, however, heterogeneous or upregulation was observed in 6/33 cases stained for LGII. These findings suggest that abnormalities in HLA class I and Class II antigen expression are frequently associated with malignant transformation of mammary epithelial cells.

CONCLUSIONS

HLA ANTIGEN EXPRESSION IN BREAST CANCER: A MULTICENTER STUDY UTILIZING FORMALIN-FIXED PARAFFINIZED TISSUES.

Despite the possible clinical significance and potential for T-cell based immunotherapy, evaluation of malignant lesions for HLA class I antigen expression is not performed routinely, even for patients who are candidates for such therapy. This reflects, at least in part, reluctance by pathologists to utilize frozen tissue sections in IHC assays. Little information is available about the usefulness of formalin-fixed paraffin-embedded tissues (FFPT) as substrates in IHC assays to evaluate tissue expression of HLA antigens. We therefore undertook a multicentric study to develop and standardize an IHC protocol using FFPTs and anti-HLA mAbs. To determine if loss of expression of MHC Class I molecules at the protein level reflect alterations at the gene level, DNA from microdissected normal and tumor tissue were evaluated with microsatellites at the MHC class I 6p21.3 locus (HLA-A, B, C determinants) and at the 15q21 beta 2 microglobulin locus for concordance of expression. HLA class I antigen down-regulation in conjunction with cellular heterogeneity of expression in three breast carcinoma cases was concordantly reported by the four participating laboratories with the anti-HLA class I antibody HC-10 and with the anti-beta 2 microglobulin L368. Furthermore, no staining of normal and malignant mammary cells was detected by the four laboratories in the lesions stained with the anti-HLA class II LGII. In contrast, infiltrating lymphocytes were strongly stained by LGII. Downregulation of class was reflected by LOH in cases 1 and 3 for the 15q21 locus and in case 1 at the 6p21 locus. The results indicate that FFPTs represent a useful substrate upon which to monitor HLA antigen expression in malignant lesions, especially when appropriate markers are used to differentiate malignant cells from lymphocytes and dendritic cells.

The manuscript is in its final revision for submission to the Journal "Tissue Antigens" (Appendix B).

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APPENDICES

A: Study Instruments
   a: Pathology Review Form: Teleform Version for Data Entry

B: Manuscripts ready for submission:
   a: HLA antigen expression in breast cancer: a multicenter study utilizing formalin-fixed paraffinized tissues
   b: Selective downregulation of HLA class I BW4 negative antigens in human leukemia suggest a combined escape from T- and NK cell attack.

1841033819

CANCER PATHOLOGY REVIEW FORM (page1)

MRN ☐☐☐☐☐☐☐

Pathology_No ☐☐☐☐☐☐☐

Path Report Date ☐☐/☐☐/☐☐☐☐

Type of tissue sample

- ○ Needle Biopsy
- ○ Excision Biopsy
- ○ Simple Mastectomy
- ○ Modified Radical Mastectomy
- ○ Other
- ○ Unknown

Stage

- ○ 0
- ○ 1 ○ 1A ○ 1B ○ 1C
- ○ 2 ○ 2A ○ 2B
- ○ 3 ○ 3A ○ 3B
- ○ 4
- ○ Unknown

Source of Breast Tissue

- ○ Left
- ○ Right
- ○ Unknown

Size of Largest Mass (cm) ☐☐.☐☐

MICROSCOPIC FINDINGS

MALIGNANCY **Present** ○ No ○ Yes

Foci ○ 1 ○ >1 ○ >multifocal

Block1 ☐☐☐ **Block2** ☐☐☐

Block3 ☐☐☐ **Block4** ☐☐☐

Block5 ☐☐☐ **Block6** ☐☐☐

Type ○ Invasive ductal ○ Invasive lobular ○ Tubular ○ Mucinous ○ Medullary ○ Inflammatory ○ Other

Carcinoma In Situ

○ No ○ Lobular ○ Ductal ○ Both

Block1 ☐☐☐ **Block2** ☐☐☐ **Block2** ☐☐☐

Grade ○ 1 ○ 2 ○ 3 **Nuclear** ○ 1 ○ 2 ○ 3

Angiolymphatic Invasion ○ No ○ Yes

Necrosis ○ No ○ Yes

Grade

Nuclear ○ 1 ○ 2 ○ 3 ○ Unknown

Tubules ○ 1 ○ 2 ○ 3 ○ Unknown

Mitotic ○ 1 ○ 2 ○ 3 ○ Unknown

Lymphnodes

Number Sampled ☐☐☐ **Number Positive** ☐☐☐

Block1 ☐☐☐ **Block2** ☐☐☐ **Block2** ☐☐☐

3374033812

CANCER PATHOLOGY REVIEW FORM(page2)

MRN

0 1 2 3 4 5 6 7 8 9

Pathology_No

0 1 2 3 4 5 6 7 8 9

ER ○ Yes ○ No

PR ○ Yes ○ No

ASSOCIATED FINDINGS

Normal Breast ○ No ○ Yes Block1 Block2 Block3

Normal Other ○ No ○ Yes Block1 Block2 Block3

Simple Adenosis (Mod-florid) ○ No ○ Yes Block1 Block2 Block3

Sclerosing Adenosis (Mod-florid) ○ No ○ Yes Block1 Block2 Block3

Apocrine Adenosis (Mod-florid) ○ No ○ Yes Block1 Block2 Block3

Hyperplasia Without Atypia (Usual Type) (Mod-florid) ○ No ○ Yes
Block1 Block2 Block3

Hyperplasia Without Atypia (ApocrineType) (Mod-florid) ○ No ○ Yes
Block1 Block2 Block3

Papilloma ○ No ○ Yes Block1 Block2 Block3

Radial Scar ○ No ○ Yes Block1 Block2 Block3

Fibroadenoma ○ No ○ Simple ○ Complex Block1 Block2 Block3

Atypical Hyperplasia ○ No ○ Lobular ○ Ductal Block1 Block2 Block3

CIS ○ No ○ Lobular ○ Ductal Block1 Block2 Block3

Reviewed by Raju ○ No ○ Yes

HLA antigen expression in breast cancer: a multicentric study utilizing formalin-fixed paraffinized tissues

M J. Worsham[1], R. Nanavati[1], U. Raju[1], S.R. Wolman[2], T. Cabrera [3], F. Garrido [3], E. A. Repasky[4], B. Hylander[4], M. Feenstra [5], M.Verdaasdonk[5], M.E.I.Schipper[5], M.Tilanus[5], S.Ferrone[4].

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Running title: HLA antigen expression in formalin-fixed tissues

Key words: HLA, antigen expression, formalin-fixed tissues, breast cancer,

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Abstract

Immunohistochemical (IHC) staining of frozen tissues has shown that abnormalities in HLA class I antigen expression are frequent in malignant cells. Despite the possible clinical significance and potential for T-cell based immunotherapy, evaluation of malignant lesions for HLA class I antigen expression is not performed routinely, even for patients who are candidates for such therapy. This reflects, at least in part, reluctance by pathologists to utilize frozen tissue sections in IHC assays. Little information is available about the usefulness of formalin-fixed paraffin-embedded tissues (FFPT) as substrates in IHC assays to evaluate tissue expression of HLA antigens. We therefore undertook a multicentric study to develop and standardize an IHC protocol using FFPTs and anti-HLA mAbs. To determine if loss of expression of MHC Class I molecules at the protein level reflect alterations at the gene level, DNA from microdissected normal and tumor tissue were evaluated with microsatellites at the MHC class I 6p21.3 locus (HLA-A, B, C determinants) and at the 15q21 beta 2 microglobulin locus for concordance of expression. HLA class I antigen down-regulation in conjunction with cellular heterogeneity of expression in three breast carcinoma cases was concordantly reported by the four participating laboratories with the anti-HLA class I antibody HC-10 and with the anti-beta 2 microglobulin L368. Furthermore, the four laboratories detected no staining of normal or malignant breast tissue for the lesions stained with the anti-HLA class II LGII. In contrast, infiltrating lymphocytes were strongly stained by LGII. Downregulation of class I was reflected by LOH in cases 1 and 3 for the 15q21 locus and in case 1 at the 6p21 locus. The results indicate that FFPTs represent a useful substrate to monitor HLA antigen expression in malignant lesions, especially when appropriate markers are used to differentiate malignant cells from lymphocytes and dendritic cells.

Introduction

HLA class I antigen expression in surgically removed malignant lesions has been extensively investigated in recent years. Frozen tissue sections have been used as a substrate in immunohistochemical reactions with mAb, since the determinants recognized by the large majority of them are not expressed in formalin fixed, paraffin embedded tissues. These studies have convincingly shown that abnormalities in HLA class I antigen expression are frequently associated with malignant transformation of cells. Furthermore studies of a limited number of patients have provided suggestive evidence that abnormalities in HLA class I antigens in malignant lesions may have a negative impact on the clinical course of the disease and on the outcome of T cell-based immunotherapy. In spite of this evidence analysis of HLA class I antigen expression in malignant lesions represents a criterion neither to evaluate patients with malignancies nor to select those to be treated with T cell-based immunotherapy. This phenomenon is likely to reflect, at least in part, pathologists' reluctance to use frozen tissue sections as a substrate in immunohistochemical reactions. To overcome this limitation in the routine analysis of HLA class I antigen expression in malignant lesions, the HLA and Cancer component of the 13th International Histocompatibility Workshop has set as one of its goals the analysis of HLA class I antigen expression in formalin fixed malignant lesions and the assessment of its clinical significance.

Distinct mechanisms have been found to underlie the multiple tumor HLA class I immuno-surveillance escape phenotypes which have been identified by immunohistochemical

staining of frozen tissue sections with mAb which recognize monomorphic, locus-specific and allele-specific determinants of HLA class I antigens. HLA haplotype loss in tumor cells has been shown to be generated frequently by total or partial deletion of chromosome 6, which carries the major histocompatibility complex in humans (24, 25). The detection of these losses has been facilitated by the recent identification of highly polymorphic microsatellite markers (short tandem repeats). Utilizing short tandem repeats mapped on chromosome 6 (6p21, HLA locus) and on chromosome 15 (5 q21 locus, β2 microglobulin), worked differences have been described in the frequency of HLA class I genes and of β2 m genes in various types of malignancies. The values range from about 15% in colorectal carcinoma lesions to about 50% in head and neck squamous cell carcinoma (26) and in cervical carcinoma lesions (27). Whether these differences reflect the use of different markers, lack of standardization in the methodology used, and/or different characteristics of the malignancies and/or patients investigated remains to be determined.

The aim of the present study was to optimize and assess the reproducibility of the assays to measure the expression of HLA class I antigens and to detect LOH at the HLA locus (6p21) and at the β2 m locus in formalin fixed, paraffin embedded malignant lesions.

Materials and Methods

Four laboratories participated in this evaluation (1-Garrido, 2-Telanus, 3- Ferrone, 4-Worsham), using shared antibodies (prepared in lab 3) and sections cut from the same tissue blocks (prepared in lab 4).

Monoclonal antibodies and conventional antisera. The anti-HLA class I heavy chain mAb HC-10, the anti-β_2 microglobulin (β_2m) mAb L368 and the anti-HLA class II mAb LGII-612.14 were developed and characterized as described **(Soldano please fill in).** Peroxidase-conjugated anti-mouse IgG xenoantibodies were purchased from Vector Laboratories, Inc (Burlingame, CA).

IHC methodology, validated in Laboratory 4 with recommended dilutions for the three antibodies was provided to the other participating centers. In brief, dilutions were as follows: mAb HC-10: 1/100 dilution, mAb L368: 1/25, mAb LGII612.14: 1/25, with overnight incubation for all three. The standardized methodology utilizes antigen-retrieval with endogenous peroxidase inactivation using 3% hydrogen peroxide, with normal horse serum as the blocking agent (Vector Laboratories Inc.). Following incubation with monoclonal dilutions, the slides are washed and incubated with biotinylated horse anti-mouse immunoglobulin G (Vector Laboratories, Inc.), followed by incubation with avidin-biotin peroxidase complex (Vectastain Elite ABC Kit: Vector Laboratories, Inc.). Immunoreactivity is visualized with 3', 3-diaminobenzidine tetrahydrochloride (Vector laboratories, Inc.) and the sections are counterstained with Mayer's Hematoxylin.

IHC Interpretation

Scoring of stain intensity in the benign lesion areas and the tumor were performed with reference to staining intensity of normal control breast tissue present either in the same
section with the breast tumor, or processed concurrently with the tumor section. Stromal lymphocytes in lymphocytic infiltrates surrounding the tissue, which stain intensely, are also considered as a reference for normal "staining intensity".

Scoring parameters included cell membrane localization. Staining interpretation is derived as follows: 0-25% of cells = negative; >25%-<50% = heterogeneous; >50%-<75% = heterogeneous; (>25%-<75% = heterogeneous);>75% = positive staining. Scoring was done by two independent reviewers in each of the four centers in a blinded fashion. Complete absence of staining as compared to the presence of staining in the normal breast epithelium is scored as "complete loss". Marked decrease in staining intensity is scored as "+", presence of some staining intensity receives a score of "++", a score of "+++" indicates intensity of stain seen in normal breast tissue or surrounding lymphocytes.

Microsatellite Marker Analysis

Processing Tumor Specimens for DNA Analysis

DNA from normal and tumor tissue was extracted from 4-5 micron sections from archival paraffin embedded tissue blocks. DNA was extracted using the QIAamp Kit (Qiagen Inc. Chatsworth, CA) according to the manufacturer's protocol.

Microsatellite analysis

A minimal microsatellite panel defining LOH at the HLA locus at 6p and the β_2m locus at 15q included D6S1618, D6S291, D6S273, D6S265 and D6S311, and D15S126 and D15S209, respectively (Table 1, Figure 1). Fluorescent-labeled primers were obtained from Perkin-Elmer Applied Biosystems (Foster City, CA). 2μl-4μ of purified paraffin DNA from a total elution volume of 200ul from normal paraffin tissue and tumor in each case was used for each PCR reaction. PCR was performed in a 1X PCR buffer (10mM Tris-HCL pH 8.3, 50 mM KCl), 2.5mM $MgCl_2$, 200 uM each dNTPs, 1uM of each primer and 0.8 units of Ampli Taq Gold polymerase (Perkin-Elmer) in a 10ul reaction volume, using a Perkin Elmer GeneAmp 9600 followed by 35 cycles, 12 min 95 °C initial denaturation, an additional 50sec denaturation at 94 °C, 60 sec annealing at 57 °C and 60 sec extension at 70 °C. Amplification was completed with a final incubation step at 72 °C for 30 minutes.

The amplified PCR products were analyzed using an automated ABI PRISM sequencer (model 310, PE Applied Biosystems), using GeneScan Rox 400 size standard (PE Applied Biosystems). In brief, 12 ul deionized formamide were combined with 0.5 ul GeneScan Rox 400 size standard (PE Applied Biosystems) and 2ul of PCR product in a Genetic Analyzer sample tube. The tubes were closed with Genetic Analyzer septa and, after short mixing (vortex), the samples were denatured in a heat block for 4 minute at 90 °C, chilled on the ice, and spun briefly in a microcentrifuge in order to collect the contents. The samples were loaded on the ABI 310 genetic Analyzer and the run in accordance with the manufacturer's protocol **(rouba)**.

Interpretation Criteria

A sample was defined as heterozygous if there were two distinct alleles for a given marker, i.e., maternal and paternal alleles were distinguishable (informative for LOH). The presence of only one size allele for a given marker, i.e., maternal and paternal alleles is indistinguishable (uninformative for LOH) was interpreted as homozygous. Loss of one of the two constitutive alleles in the tumor was scored as loss of heterozygosity (LOH). LOH was assigned when the fluorescent signal of one of two alleles was reduced by more than 30% as compared with the heterozygous control normal DNA sample.

LOH was defined mathematically as follows:

Height of tumor allele two/Height of tumor allele one

Height of normal allele two/Height of normal allele one

To illustrate, in case 1 for markersD15S209:

1) Size of alleles: 197.86 (allele one or shorter) and 203.73 (allele two or taller) in the normal DNA sample;
   197.87 (allele one) and 203.69 (allele two) in tumor sample.
2) Peak height: 550 (allele one) and 256 (allele two) in normal sample; 463 (allele one) and 525 (allele two) in tumor sample

Calculations

Height of tumor allele two (=526/height of tumor allele one (=463) =0.40

Height of normal allele two (=256)/height of normal allele one (=550)

or the inverse (1/x=2.47) that indicates a reduction in the longer allele of the tumor by more than 40% (60% exactly).

The calculations for peak areas were identical.

RESULTS

Summary of Validation Study Results

Laboratory 2 employed a negative control and followed the provided protocol and dilutions exactly. Laboratory 1 made several variations to the provided protocol. Changes included the following: no avidin/biotin block, antigen retrieval was accomplished with the vector antigen retrieval solution in the microwave, DAB was used as a chromagen instead of AEC, and CD45 staining was also done to confirm the presence of lymphocytes and dendritic cells in tumor infiltrates.

Results

HLA expression

In Case 1, for HC-10, among the 4 laboratories, expression was concordantly noted as heterogeneous in the tumor (Table 2). Heterogeneity of staining was attributed to varying degrees of tumor differentiation and also due to the presence of heavy lymphocytic infiltrate and CD45-positive dendritic cells within tumor areas, which resulted in an increased staining intensity in the tumor when compared with the normal/benign epithelium. For L-368, normal ducts were scored as negative for Case 1 by Laboratories 1 and 2 and heterogenous by Laboratories 3 and 4. For the tumor, when compared to staining intensities of stromal and lymphoctyic cells, all 4 laboratories noted downregulation for L-368. With LGII, agreement among the four centers was complete.

For case 2, for HC10, Laboratories 1, 2 and 3 scored normal epithelium as positive, with a heterogeneous interpretation by Laboratory 4 depending upon degree of tumor differentiation. All four laboratories scored tumor areas as negative. For L368, laboratories 2, 3 and 4 had positive staining in normal ducts. Laboratory 1 reported weak staining in normal ducts with L368. Within tumor areas, all four laboratories indicated loss of staining for L368 with heterogeneity of staining in more well-differentiated areas of the tumor. While there was complete concordance for lack of staining with LGII, Laboratory 1 noted some staining within the luminal epithelium of the normal ducts.

In case 3, there was concordance for staining of the tumor areas with all 3 antibodies among the four centers. Tumor areas for HC10, L368 and LGII were negative in undifferentiated areas of the tumor, with heterogeneous staining in the well- differentiated areas (25%) for HC10 and L368. Normal breast epithelium was scored as weakly staining by Laboratory 1. Normal breast epithelium was not present in the tissue sections examined by Laboratory 2, 3, and 4. Stromal cells were present and scored as positive among all four laboratories.

The quality of the staining and the overall results were similar despite variations to the protocol. Microwave and citrate buffer antigen-retrieval substitutions yielded comparable results. Laboratory 2 demonstrated that a negative control is useful as an internal standard for reagent optimization and for noting any non-specific reactivity. CD45 staining as employed in Laboratory 1 may be helpful in identifying the infiltrating lymphocytes in germinal centers and dendritic cells as seen in case 1.

Microsatellite Analysis

Lab 1, Lab 2 and Lab 4 performed Microsatellite analysis. Loss of heterozygosity was observed at the β2 microglobulin locus in Case 1 and 3, concordant with down regulation at the level of HLA expression. This locus was uninformative in Case 2. For the HLA class I loci at 6p21, LOH for three 6p21 markers suggested concordance with down regulation of class I antibodies, HC-10 and L3682 in case 1. For cases 2, 6p21markers were either uninformative, normal or did not yield an interpretable result. For case 3, 6p21 markers indicated LOH for D6S105 and MSI for maker marker D6S265 by Lab 4, as compared to normal and uninformative results, respectively, by Lab 1 (Table 3).

For an interpretation of LOH, there was concordance, except for marker D6S105 in Case 1, which was scored as LOH by Lab 1 and 4, and homozygous by Lab 2. In case 3, LOH for D6S105 and MSI for maker marker D6S265 by Lab 3 were scored as normal and uninformative respectively, by Lab 1. Among laboratories, for case 1, all three indicated LOH for markers D6S265 and D6S276, and LOH for D15S209 by Lab 1 and Lab 4. For all three cases, lab 1 and Lab 4 concordantly interpreted marker D6S1618 as homozygous

(uninformative). Lab 1 and 4 noted homozygosity for marker D15S126 in case 1; Lab 2 did not generate an interpretable result.

Discrepant results were noted in Case 2, for marker DS105; Lab 1 indicated heterozygozity in the normal and tumor, whereas Lab 2 and Lab 4 indicated a homozygous result. This was also the case for marker D15S209 in case 2, which was scored as heterozygous normal by Lab 1 and homozygous by lab 4. In case 3, LOH was indicated by Lab 4 for marker D6S105, and microsatellite instability (gain of an allele as compared to a homozygous status in the normal) for D6S276, an interpretation that was not shared by Lab 1.

Discussion

Immunohistochemical (IHC) staining of frozen sections of malignant tumors has shown that abnormalities in HLA class I antigen expression are frequent in malignant cells. Despite the possible clinical significance and potential for T cell-based immuno-therapy, evaluation of malignant lesions for HLA class I antigen expression is not performed routinely, even for patients who are candidates for such therapy. This reflects, at least in part, reluctance by pathologists to utilize frozen tissue sections in IHC assays.

The potential predictive value of HLA expression in evaluation and therapy planning has been under-utilized. The importance of this marker in the evaluation of breast cancer patients prompted our undertaking of a multicentric study approach to develop and standardize an IHC protocol using formalin-fixed, paraffin-embedded tissue and anti-HLA mAbs. HC-10, a mAb to a determinant expressed on β2-microglobiulin free HLA class I heavy chains (chromosome 6p21) and L368, an anti-human β2- microglobulin (chromosome 15q21) were evaluated in sections of archival formalin-fixed paraffin embedded breast tumors.

HLA class I antigen downregulation in conjunction with and without heterogeneity was concordantly scored in tumor regions of all cases with HC10 and mAb L368. Furthermore, no staining of either normal or malignant breast tissue was detected by the four laboratories in lesions stained with LGII, although lymphocytes in the same section were strongly stained. While there was some lack of agreement with staining interpretations in normal breast epithelium for L368, lymphocytes and stromal cells were judged positively stained by all four laboratories.

DNA analysis from archival tissue permits retrospective characterization of disease and has facilitated molecular data gathering from large population-based epidemiological study cohorts. It can also serve as surrogate tissue material for genetic testing of diseases or syndromes in the absence of fresh or frozen tissue or peripheral blood lymphocytes. In retrospective studies for DNA marker characteristics, formal in-fixed tissue is mostly available in paraffin blocks. For the detection of allelic imbalance in tumor tissue DNA from paired normal and tumor tissue is necessary. Lack of or scarcity of normal or tumor tissue in the paraffin sections or poor quality of the DNA due to the use in the past of unbuffered formaldehyde during the fixation process presents serious limitations to studies that rely on archival patient resources.

Discordant results and lack of interpretable results in some instances reflect in part low DNA yields and poor quality DNA that become factors in interpretation of low signal allele peaks, stutter peaks and difficulty with amplification of larger size alleles due to degradation of DNA inherent in formalin-fixed archival tissue sources. For this study,

limited resources that required a four-way distribution hampered availability of DNA for repeat sampling in some cases. Despite these challenges, for markers D6S276 and D6265, an interpretation of LOH was validated by all three participating centers, and D15S209 by at least two laboratories.

The results indicate that formalin fixed paraffin embedded tissue represent a useful substrate to monitor HLA antigen expression in malignant lesions, especially when appropriate markers are also used to differentiate malignant cells from lymphocytes and dendritic cells. Loss of heterozygosity for markers representative of the HLA loci at 6p21 and 15q21 agreed with down regulation of expression of class 1 gene expression. LOH associated with loss of the HLA locus at chromosome 6p supports an extended mechanism that generates HLA haplotype loss previously described in different histological tumor types (refs). Contaminating stroma can mask LOH results and microscopic or laser microdissection, to separate stroma and tumor would facilitate an interpretation of LOH in those cases with unclear results.

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Figure 1: A: Normal breast epithelium from case 1 illustrating uniform membrane "+ + + +" stain intensity with HC-10. B: Concurrent invasion lesion from Case 1 showing reduced expression of HC-10. Note stromal cells and scattered lymphocytes with "+ + + +" staining intensity.

Table 1 **HLA Antibodies**

HLA Class I	HLA Class II	Other
HC-10	LGII	CD45
L368		

Table 2 **Summary of IHC Validation Results**

Results	Case 1								
Antibody	HC-10		Outcome	L368		Outcome	LGII		Outcome
	Normal	Tumor		Normal	Tumor		Normal	Tumor	
Lab 1	‡	‡	loss	‡	‡	loss	‡	‡	normal
Lab 2	‡	‡	loss	‡	‡	loss	‡	‡	normal
Lab 3	‡	‡	loss	†*	‡	loss	‡	‡	normal
Lab 4	‡	‡	loss	†	‡	loss	‡	‡	normal

Results	Case 2								
Antibody	HC-10		Outcome	L368		Outcome	LGII		Outcome
	Normal	Tumor		Normal	Tumor		Normal	Tumor	
Lab 1	‡	‡	loss	†*	‡	loss	‡*	‡	normal
Lab 2	‡	‡	loss	‡	‡	loss	‡	‡	normal
Lab 3	‡	‡	loss	‡	‡	loss	‡	‡	normal
Lab 4	†*	‡	loss	‡	‡	loss	‡	‡	normal

Results	Case 3								
Antibody	HC-10		Outcome	L368		Outcome	LGII		Outcome
	Normal	Tumor		Normal	Tumor		Normal	Tumor	
Lab 1	‡	‡	loss	†*	‡	loss	‡	‡	normal
Lab 2	‡	‡	loss	‡**	‡	loss	‡	‡	normal
Lab 3	‡	‡	loss	‡**	‡	loss	‡	‡	normal
Lab 4	‡	‡	loss	‡**	‡	loss	‡	‡	normal

‡= complete concordance
†= incomplete concordance
‡* = complete concordance, see text
†* = incomplete concordance, see text
‡** = stromal cells and lymphocytes

Table3 **Summary of Loss of Heterozygosity Validation Results**

Lab 1=Garrido, Lab2 = Telanus, Lab 3= Ferrone, Lab 4= Worsham

Table

Markers	Case 1 (S93-16818)						Case 2 (S94-33689)						Case 3 (S95-3416)					
	Lab 1		Lab 2		Lab 4		Lab 1		Lab 2		Lab 4		Lab 1		Lab 2		Lab 4	
	N	T	N	T	N	T	N	T	N	T	N	T	N	T	N	T	N	T
D6S1618	nr	h	nd	nd	h	h	**h**	**H**	nr	nr	**h**	**h**	**h**	**h**	nr	nr	**h**	**h**
D6S291	nr	ht	nd	nd	nr	ht	nr	Nr	nr	nr	nr	h	nr	nr	nr	nr	nr	?ht
D6S273	**ht**	**loh**	nr	nr	**ht**	**loh**	h	H	nr	nr	nr	h	ht	ht	nr	nr	nr	ht
D6S265	**ht**	**loh**	**ht**	**loh**	**Ht**	**loh**	ht	Ht	nr	nr	nr	?ht	ht	ht	nr	nr	nr	ht
D6S105	**ht**	**loh**	***h***	***h***	**ht**	**loh**	***ht***	***Ht***	***h***	***h***	***h***	***h***	***ht***	***ht***	nr	nr	***ht***	***loh***
D6S276	**ht**	**loh**	**ht**	**loh**	**ht**	**loh**	**h**	**H**	nr	nr	**h**	**h**	***h***	***h***	nr	nr	***h***	***ht***
D6S311	**h**	**h**	nr	nr	**h**	**h**	nr	Nr	nr	nr	nr	nr	nr	nr	nr	nr	nr	nr
D15S126	**h**	**h**	nr	nr	**h**	**h**	nr	Nr	nr	nr	h	h	nr	nr	nr	nr	ht	loh
D15S209	**ht**	**loh**	nr	nr	**Ht**	**loh**	***ht***	***Ht***	nr	nr	***h***	***h***	ht	nr	nr	nr	ht	loh

nr= no result; h= homozygosity; ht=heterozygozity; LOH= loss of heterozygozity

Selective downregulation of HLA class I BW4 negative antigens in human leukemia suggest a combined escape from T- and NK cell attack.

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Abbreviations:

ALL: acute lymphoblastic leukemia
AML: acute myeloid leukemia
CLL: chronic lymphoblastic leukemia
HLA: human leukocyte antigen
NK: Natural Killer cell
CDC: complement dependent cytotoxicity
MFI: mean fluorescence index

Keywords: HLA class I, leukemia, downregulation, Bw4, Bw6, Natural Killer cell, immunosurveillance

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Introduction

The classical HLA class I molecules (HLA-A, HLA-B, and HLA-C) are expressed on the surface of most nucleated cells as a heterodimer composed of a 45 kDa polymorphic heavy chain (α-chain) and a 12 kDa monomorphic light chain (β2-microglobulin), presenting an 8 to 11 amino acid peptide to cytotoxic/cytolytic CD8 expressing T-lymphocytes. Every individual expresses two alleles for each locus for a total of six alleles. These cells are triggered to kill virus-infected cells or tumor cells presenting cytosolically degraded foreign viral or tumoral peptides on class I molecules. HLA class I molecules also interact with receptors on natural killer (NK) cells. They are equipped with a set of inhibitory and activatory killer immunoglobulin-like receptors that specifically recognize groups of HLA-A, -B, or -C alleles (1). When the predominant inhibitory signal on NK cells is lost due to downregulation or loss of one or more HLA class I alleles the NK cell becomes activated and will eliminate the viral-infected or malignant cell. Accordingly, downregulation or loss of HLA class I expression would not only lead to impaired T-cell recognition and escape of T-cell immunity but should also make these cells susceptible to NK attack.

Down-regulation of HLA class I expression seems to be a common event in solid tumors with various underlying mechanisms (2,3). Reduced expression has been observed in basal cell, breast-, bladder-, cervical-, colorectal-, head and neck-, laryngeal-, oesophageal-, lung-, renal cell- and pancreas carcinomas (4-13, 14, 15, 16) as well as in neuroblastoma, melanoma, prostate cancer and other tumor types (17-19, 20).

According to these studies on solid tumors 4 major types of changed HLA class I expression were recognized: 1) total loss of HLA-molecules, 2) haplotype loss, 3) HLA locus loss and 4) allele loss or a combination of these (2). Thus, it appeared that important changes occurred allowing the tumor cells to escape from cytolytic/cytotoxic T-cell activity. Because these studies were nearly exclusively done on solid tumors with immunohistochemistry techniques only a limited idea on quantitative changes of HLA molecules could be gathered.

The interest of studying HLA expression in solid tumors was mainly induced by the discovery of tumor specific tumor antigens (melanoma), the concomitant start of peptide vaccination protocols and the need for selection of patients. Indeed, eligible patients entering these vaccination studies should be positive for the restrictive peptide-binding HLA determinant on the tumor cell.

During the last years numerous leukemia-specific translocations or overexpressed genes were described both in myeloid and lymphoid leukemia's that could eventually serve as tumor specific antigen in leukemia vaccination programs (21). A natural or induced immune response against some of these translocations or genetic abnormalities was already described in several reports (22,23).

Over time however, only a few limited studies on HLA class I expression have been done on hematological malignancies such as in B cell and Hodgkin lymphoma's (24,25), CLL (26), ALL and AML (27-29). Total lack of HLA class I molecules in non-Hodgkin lymphoma has been associated with an aggressive clinical course (30). In AML, Savoia et al (31) reported an increased leukocyte concentration at diagnosis with loss of HLA class I molecules.

The aim of the present study was to map the HLA class I abnormalities on acute and chronic leukemic cells and to compare the results with those of normal patient or donor cells. Therefore we used both qualitative and quantitative techniques involving serology, flow cytometry and molecular biology. Moreover, a series of allospecific human monoclonal antibodies was extensively tested and used to study HLA expression at the allelic level.

Materials and methods

Samples

Leukemia patients

EDTA anticoagulated blood or bone marrow samples were obtained from 64 leukemia patients at diagnosis. The only selection criterion was CD19+ of CD33+ membrane expression for lymphoid or myeloid tumors respectively. All patients' samples were obtained from the Clinical Hematology Department of the Academic Hospital of the Vrije Universiteit Brussel, Brussels and the Université Catholique de Louvain, Brussels, Belgium.

Control individuals

Normal blood or bone marrow was obtained from regular blood donors or from allogeneic bone marrow donors at the time of collection. The institutional Ethics Committee approved this study.

These samples were used to isolate normal, cellular counterparts to serve as reference material for the flow cytometry studies.

Mononuclear cells from peripheral blood and bone marrow were separated by Ficoll-Hypaque density gradient centrifugation. The isolated cells were further washed and diluted in PBS Facsflow (Becton Dickinson, San Jose, CA, USA) supplemented with 0.5% bovine serum albumin.

Magnetic cell sorting system (MACS)

`The MACS-system and the microbeads were obtained from Miltenyi (Sanvertech, Boechout, Belgium) and applied as described by the manufacturer. In brief, a starting population of mononuclear cells was incubated for 15 min with the appropriate mouse anti-CD antibodies (see further). The cells were washed in buffer and incubated in 100µL of a 1:10 dilution of the anti-mouse labeled magnetic microbeads for 15 minutes on ice.

The cell suspension was then applied to a MIDIMACS column in a magnetic collar. The column was washed thrice to obtain the untouched cells. The bound cells (touched fraction) were recovered by removing the magnetic collar and passing 5 mL of buffer through the column using a plunger.

It was decided at the start of the study that all HLA-expression experiments would be done on negative (untouched) isolated cell suspensions, primarily to exclude the possible interference from antibody-coated magnetic beads on cells in further procedures and to examine the HLA expression on the tumor cells and normal cells from a patient in identical conditions.

Therefore, two individual magnetic bead separations were performed on each peripheral blood or bone marrow sample to obtain untouched normal or tumor cell fractions. In addition, different microbeads were used depending on the type of tumor.

To isolate tumoral B cells from CLL-B or ALL-B samples the patients normal cells were magnetically labeled by a cocktail of anti-CD2, -CD4, -CD11b,-CD16 and -CD36 antibodies. The unlabeled CD19+ B cells were recovered by simple washing. In a second procedure the patient's normal cells were obtained after the tumoral B cells were retained on the column by anti-CD19 microbeads. In the case of myeloid tumors the patient's normal cells were isolated after the tumor cells were bound to the column through anti-CD33 microbeads. Untouched CD33+ tumor cells were obtained by retaining the normal cells on the column by a combination of anti-CD2 and anti-CD19 microbeads. The purity of the isolated cells, as tested by flow cytometry, was always higher than 95 %.

All touched, positive fractions (patient tumor- or normal cells) were cryopreserved in complete medium containing 10% DMSO for eventual DNA isolation purposes.

An identical procedure was used to isolate normal B-cells and normal T-cells from peripheral blood or bone marrow from healthy donors.

No attempt was made in this study to isolate the small fraction of NK cells (5%) out of the major T-cell fraction (95%). These cells are further called "normal cells" in the study.

The purified, untouched cells were preferentially used for general flow cytometric purposes. The remaining cells were further used for complement-dependent cytotoxicity assays and eventually cryopreserved for later on staining with allele specific mAb's.

Flow cytometry

The following unlabelled, mouse mAb's against differentiation antigens were used for leukemic cell type assignment: anti-CD2 (clone leu 5b), anti-CD3 (clone leu 4), anti-CD5 (clone leu1), anti-CD16 (clone leu 11), anti-CD19 (clone leu 12), anti-CD45 (leuco) and anti-CD56 (clone leu19) were obtained from Becton Dickinson. Anti-CD13 (clone My7) and anti-CD33 (clone My9) were obtained from Analis (Namur, Belgium).

A second panel of mouse anti human mAb's was directed against general HLA antigens. Anti-locus A (clone LGIII-147-4) and anti-β2 microglobulin (clone NAMB-1) were obtained from Prof. S. Ferrone and anti-locus B+C (clone B1.23.2) from Dr. A. Mulder. Anti HLA total

class I (clone W6/32) and anti HLA total class II (clone Tu-39) were obtained from ATCC. Anti HLA locus DR (clone HLA-DR) was obtained from Becton Dickinson.

In addition, a panel of human mAb's (Dr. A. Mulder) recognizing single HLA alleles were used to study individual allospecific expression levels in normal healthy individuals and leukemic patients and to confirm allelic losses found by cytotoxicity assays.

Cell suspensions of 4 x 10^6 cells/mL were made in PBS supplemented with 5% BSA. Twenty-five µL of this suspension was incubated for 20 min with 25 µL of each mAb at room temperature. The cells were rinsed with PBS + 1% BSA.

All mouse antibodies were detected with F(ab')2 fragments of goat anti-mouse isotype specific antibodies coupled to fluorescein isothyocyanate (FITC). Human allele-specific monoclonal antibodies were detected with F(ab')2 fragments of rabbit anti-human isotype specific antibodies coupled to FITC. In each case, at least two mAb's against single HLA antigens, not present on the patient's cells, were used as negative controls. All secondary reagents were obtained from Prosan (Merelbeke, Belgium).

After washing 500 µL of FACS flow solution was added (Becton Dickinson) and the samples examined consecutively. Data were acquired, analyzed and displayed by a Coulter Epics II – MCC cytometer using the Coulter System II – software v 3.0 (Coulter, Miami, Florida, USA). The mean fluorescence intensity (MFI) from samples was noted and used for further analysis.

HLA typing

Serological HLA typing

The HLA-A and HLA-B phenotype was established on the different isolated cell fractions using the conventional complement-mediated microlymphocytotoxicity test (CDC-testing). Each plate contained at least two sera identifying individual HLA-antigens. In individual cases (weak or extra reactions) other commercially available, serological plates were used (Biotest Seralc, Kortenberg, Belgium). Allelic losses were considered when no reaction was observed on the tumor cells compared to the patient's normal cells.

All microcytotoxicity assays were supervised by the same experienced technician ensuring reproducibility and reliability of the assays during the study.

Molecular HLA typing

Reverse hybridization technique

In all cases were locus A or B showed homozygosity by serology, molecular typing was performed based on the reverse hybridization technique (Innogenetics, Ghent, Belgium).

DNA was isolated using a routine salting-out method (DNA E-Z Prepkit, Orchid Diagnostics Europe, St Katelijne Waver, Belgium).

Sequence based typing

In cases were complete HLA-losses on tumor cells were detected by serology, DNA was extracted from cryopreserved normal and leukemic cells (touched fractions).

First, a 2 KB PCR amplicon spanning exons 1 to 5 from locus A and B was generated using a Gene Amp PCR 9700 (Applied Biosystems, Foster City, Ca, USA). The PCR product was always cleaned-up using Centricon YM-100 filters (Millipore, Brussels, Belgium) and visualized in agarose gel electrophoresis using ethidiumbromide to show the single 2 KB band before sequencing.

Next, sequencing reactions were performed separately for exons 2, 3 and 4 both in forward and reverse directions for each sample using Big Dye Terminator Chemistry (Applied Biosystems). Sample electrophoresis was done on an ABI Prism 310 sequencer. Sequencing reactions were analyzed using ABI Match Tools PPC v 1.0. Both the PCR amplification and sequencing reagents were obtained from Applied Biosystems.

Cell lines

The following human leukemic cell lines molecular typed for HLA class I, were used in testing the specificity and reactivity of the human mAb's: MONO-MAC-6 (A03, BO7, B51), NALM-6 (A01, A02, B08, B15), RPMI-8402 (A01, A29, B07, B38), ML-2 (A02, B44, B51), NB-4 (A11, B35, B40), 697 (A02, A25, B07, B15), KARPAS-299 (A03, A11, B07, B35) and K562 (A11, A31, B18, B40).

Statistics

The non-parametric (two-sided) Mann-Whitney U test was used to calculate probabilities between groups. $P \leq 0.05$ was considered significant.

Results

Reactivity and specificity of the human allele-specific antibodies

Before using the allele-specific human mAb's in our study they were first extensively tested for reactivity and specificity on HLA class I typed donors and cell lines. The results are shown separately for HLA locus A (Table 1) and locus B (Table 2). The reactivities of the Ab's tested in flow cytometry always showed expression levels ranging from 1 till 3 logs stronger than the negative controls. The results for the NALM-6 and MONO-MAC-6 cell lines are shown (Fig 1a,b). Overlapping profiles were never observed. Some antibodies did react with an epitope present on distinct HLA antigens. For example, clone SN66E3 reacted with A2 but also with A28. In these cases, the donors or cell lines were selected for having only one of these antigens. Other mAb's, such as clone VDK1D12, are restricted in the recognition of only one HLA antigen. The overall results showed that all mAb's, except clone BR011F6, reacted as predicted by the HLA type of all the cells under study confirming there specificity. Clone BR011F6 did not recognize A1104. The HLA class I negative K562 cell line remained negative with all relevant mAb's (not shown).

Locus-specific analysis of HLA antigens

In a first attempt to detect HLA expression abnormalities 46 leukemic patients, including CLL-B (n=17), ALL-B (n=9) and AML (n=20), have been studied with common anti-HLA mAb's. The expression level of the untouched leukemic B cells was compared to the expression on normal B cells from healthy donors. In CLL-B total class I, as measured by the W6/32 mAb, was significantly lower than on normal B cells (Table 3). No significant difference was however noted for locus A or B. Total HLA class II was also significantly lower on the tumoral B cells.

In ALL-B the decrease in total HLA class I was even more pronounced than in CLL-B. In all individual cases (data not shown) a severe decrease for locus A and B was observed. No

significant changes were noted for class II expression. Further more, all CLL-B and ALL-B cases showed β2 microglobulin expression.

In contrast, in CD33+ AML cells total levels of class I and II were increased when compared to normal $CD33^+$ bone marrow derived progenitors (Table 4). Significance could however not be shown for individual locus A or B. Individual data (not shown) revealed that only two cases showed a moderate decrease in total HLA class I expression. All showed expression of β2 microglobulin. Expression of total HLA class II, and more specially locus DR, was increased.

Detection of HLA class I total loss phenotypes

Immunomagnetically separated normal and tumor cells from 37 leukemic cases were analyzed by CDC (Table 5). The high purity and general HLA class I expression of two representative cases is shown (Fig 2a,b). In 5 cases complete HLA losses were detected. One patient (case 1) showed a complete locus A loss and four others showed a complete loss of one HLA-A (case 29 and 38) or HLA-B (case 7 and 44) allele.

In a number of cases to few normal cells could be isolated to allow serological typing. To avoid false conclusions on allelic losses, the HLA homozygosity of case 21 was therefore confirmed on the tumor cells by molecular typing.

Confirmation of HLA-A or -B allelic losses by flow cytometry.

Monoclonal antibodies against single HLA alleles allow the study of HLA antigens by flow cytometry. Cryopreserved patients normal and tumor cells were available from 4 cases were a complete single allele loss was detected by CDC. Allele-specific mAb's were available against all HLA-A and –B antigens form the 4 cases. Figure 3 shows that the expression of HLA A10 and A31 in case 1, B8 in case 7, HLA A28 in case 29 HLA A1 in case 38 are completely negative. The contour plots are identical to those from the negative isotype controls. On the other hand HLA-B8 (case 38) expression is nearly negative on flow but scored positive in CDC. This illustrates that the CDC serological HLA class I testing is extremely sensitive and is therefore only useful in detection of complete allelic or locus losses and not for incomplete downregulations. The expression of HLA B8 is approximately

10-times lower (1 log) on the tumor cells than on the patients normal cells. On the same tumor it can be observed that a small fraction of the tumor cells didn't express HLA A2 anymore. This could be an example of an emerging variant tumor clone. HLA total losses were never detected on the patient's normal cells illustrating the relation of HLA losses with tumor cells.

Mutation analysis

In an attempt to explain the HLA allele losses on the tumor cells the HLA-A (cases 1, 29, 38) or -B (case 7) genes were sequenced in forward and reverse direction for exons 2, 3 and 4 from the tumorcells and the patients normal cells. The results for all cases on both cell fractions were completely identical excluding a mutational problem for the exons under study. The β2-microglobulin gene was not sequenced since other HLA class I alleles on the tumor cells were present on the cell's surface excluding a mutation, or transcriptional problem of the β2-microglobulin gene.

Allele-specific expression levels on leukemic cells

During this part of the study the expression levels of individual alleles were studied on 32 leukemic samples using previously well characterized human mAb's. The expression levels were compared to those obtained from the patients normal cells and not to those from unrelated donors. In our opinion is comparison at the allelic level indeed more appropriate and accurate with cells having the same set of HLA alleles. Indeed, differential expression of HLA-A and –B antigens is genetically predetermined and inherited according to Mendelian laws (32). A drawback is that these cells are not identical cellular counterparts.

The results for the tumor cells and the patient's normal cells are given expressed as MFI and presented separately for locus A and B (Table 7). A ratio R was than calculated (R= MFI tumor cell / MFI T cells). We concluded for downregulation if the R-value was lower than 1. Data on the quantitative expression of individual alleles in healthy individuals are scarce in the literature. During the characterization of the human mAb's, individual analysis of normal T and B cells allowed us to investigate this point. In normal, healthy individuals expression of total HLA class I was always higher on B cells than on T cells (n=10; data not shown).

Similar to total HLA class I levels, individual expression levels of locus A or B were higher on B than on T cells. No single exception was observed. The mean MFI value was approximately double on B cells than on T cells. These results were confirmed very recently (33).

For the HLA-A locus, 56 alleles could be studied in 32 patients and according to our definition 20 alleles showed a downregulated expression (35 %). For the HLA-B locus 50 alleles were studied and 19 showed a downregulation (38 %). Thus, a total number of 106 alleles were studied. Combining the locus A and B results makes, that in 21 out of the 32 patients (65 %), one or more allelic downregulation(s) were observed.

Some HLA-A and -B antigens could not be studied because the relevant antibody was lacking. Locus C was also not studied. Thus, it is possible that the exact number of patients with a downregulation on the tumor cells of HLA locus A, -B, -C or a combination of these would even be higher. The patients that showed a complete allelic loss on the tumor cells and described earlier, were not included here.

Downregulation according to the presence of the Bw4 or Bw6 epitope

Allelic downregulation was therefore further analyzed according to the presence or absence of the Bw4 or Bw6 epitope (34). From the 36 HLA-B alleles expressing Bw6, 16 showed downregulation (44 %). In contrast, on the 21 HLA-A and -B alleles having the Bw4 epitope, only 3 showed a downregulation (14%). Fourty % (20/49) of the BW4/BW6 double-negative antigens also showed downregulation. The difference in frequency in downregulation of alleles expressing Bw4 or Bw6 is statistically significant ($p<0.03$). Since Bw4-positive HLA class I antigen expression is under the control of NK cells these results suggest that preferentially those alleles are downregulated that are not under NK cell control. This finding suggests that emerging tumor cells, by downregulating HLA class I alleles (but not BW4-positive antigens) not only escape from T-cell but also from NK killing.

Discussion

Our first objective was to investigate whether HLA class I alterations, such as those observed in solid tumors were detectable in leukemia. In this study HLA expression on leukemic cells

was compared to donor-derived normal cellular counterparts (first part of the study) or to the patients own remaining normal cells (second part). This methodology allowed comparison of identical cells but equipped with a different set of HLA antigens versus non-identical counterparts but completely HLA matched cells. Throughout the study we only used untouched, immunomagnetically purified leukemic or normal cells. This was mainly to exclude any interference with antibody coated magnetic beads or it was necessary for the technique itself. Indeed, CDC analysis on the leukemic cells would score positive, even with an HLA class I loss, if normal cells would grossly contaminate the leukemic test sample.

In a first part of the study we investigated by flow cytometry the general expression of HLA class I on leukemic subtypes by measuring total class I, locus A, locus B and β2 microglobulin. This revealed a decrease of total HLA class I in CLL-B and ALL-B. A pronounced HLA-A and-B locus-specific decrease was especially demonstrated in ALL-B. Expression of β2 microglobulin was detected in all leukemic samples excluding a total HLA class I loss as described in solid tumors (35). Additionally, HLA class II was decreased in CLL-B, but not in ALL-B.

In contrast, malignant CD33+ AML cells show an upregulated class I and II expression when compared to normal cellular counterparts. In agreement with the study of Wetzler et al (28) abnormalities in HLA class I are infrequent in AML, but it was surprising that in AML the phenomenon of overexpression could co-exist with a complete absence of a single allele (case 29 and 44). Indeed, a complete allelic loss was only detected in 5 out of 35 (13%) leukemic cases by CDC (Table 5).

The AML and CLL samples with a complete allelic loss were further analysed, but mutation analysis of the polymorphic regions of locus A or -B antigens did not reveal any abnormality. These results are in agreement with those from Brouwer et al (27). They also didn't detect HLA class I genetic abnormalities. Moreover, they could restore HLA class I allelic expression by interferon-γ treatment and subsequent T-cell recognition and lysis of the target cells. Real et al (35) also showed that suppression of single alleles could be restored by cytokine treatment. Further DNA or RNA- based analysis could however not be done in our

study due to the lack of sufficient material. A new bloodsample could not be obtained since all patients with a complete allelic loss died very soon after diagnosis.

In the second part of the study the expression of individual alleles was assessed with the use of highly specific mAb's. The comparison of the allelic expression on the leukemic cells and on the patient's normal cells made a detailed analysis possible. On 32 leukemic samples 116 individual allelic expression profiles could be studied. Downregulation was observed in 35 % and 38 % of HLA-A and HLA-B alleles respectively. This means that in the majority of the leukemic patients (65%) downregulation was observed for the HLA-A and/or HLA-B locus. HLA-B has been divided by serology into two groups: Bw4 and Bw6. In contrast to HLA-C, only one group of HLA-B allotypes is involved in inhibition of NK cells. Whereas HLA-B allotypes that carry the Bw4 serological epitope provide protection from NK attack, those with the Bw6 epitope do not. Further study of our leukemic samples made clear that preferentially Bw6 positive and non-Bw4/non-Bw6 containing alleles were the subject of downregulation. In solid tumors, other investigators also noticed the preferential downregulation of the HLA-B locus or B alleles but it was apparently never analyzed according to the Bw4 and Bw6 allotypes (37,38).

In conclusion, in leukemic samples a complete allelic loss was seen in a modest number of samples (10 %) but downregulation seems to be a common event in leukemia. In our opinion downregulation will finally end-up in a complete allelic loss during tumor progression. All the patients that we detected with a complete allelic loss quickly died after diagnosis suggesting a higher degree of malignant behavior of these tumors. Moreover, this could be one of the reasons why a total loss is rather infrequently detected during our and other studies (27). It is tempting to speculate that this high degree of downregulation is the result of selective pressure due to an upcoming natural anti-leukemic T-cell immunity. This activity was already described by others (39). If evasion of T-cell immunosurveillance is the case, than the escaping leukemic clone, by downregulating HLA class I antigens, should or could become a target for NK attack unless the relevant NK receptors are not available in the patients repertoire, NK receptor expression is disturbed (40) or downregulation is limited to those

alleles that are not under the control of autologous NK cells. These are HLA-A alleles not expressing the Bw4 epitope (none-Bw4/none-Bw6) and HLA-B Bw6 positive alleles. Together our findings point to the conclusion that leukemic cells, especially those of lymphoid origin, developed strategies to escape from both T-cell and NK-cell attack.

If preferential downregulation of non-Bw4 class I alleles proves to be a general phenomenon also observed in solid tumors, than vaccination protocols should preferentially include tumor-specific peptides that can bind to Bw4 containing alleles. An eventual escape from T-cell immunity would render them susceptible to autologous NK cells.

Total HLA class I loss, haplotype loss or locus loss are frequently seen in various solid tumors but not in leukemia. From our study allelic downregulation seems to be the most common abnormality in human leukemia. Therefore, future studies on these malignant cells should preferentially include reagents able to dissect expression at the allelic level (allele-specific antibodies) over those to backbone structures (anti-locus A and B, β2m, total anti-class I).

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Legends figures and tables

Figure 1. HLA expression on cell lines

Flow cytometric histograms of HLA class I and II antigens on the NALM-6 (Figure 1a) and MONO-MAC-6 (Figure 1b) cell lines as detected by different specific mAb's described in materials and methods. Fluorescence intensity with anti-HLA mAb's (black) was compared to that of irrelevant, isotype-matched controls (white).

Figure 2. HLA class I expression on immunomagnetic isolated malignant and normal cells.

In Figure 2a untouched, normal cells were incubated with anti-CD3 (black) and anti-CD19 (white) (panel A), with anti-locus A (white), anti locus-B (black) or control mAb (dotted) (panel B), and anti-W6/32 (black) or control mAb (white) (panel C). The untouched malignant cells were incubated with anti-CD3 (white), anti-CD19 (black) (panel D), with anti-locus A (white), anti-locus B (black) or with control mAb (dotted) (panel E) and anti-W6/32 (black) or control mAb (white) (panel F). The expression levels of HLA locus A and B and total class I are lower on the malignant cells than on the patients normal cells.

Figure 2b shows the results on the leukemic cells and normal cells of an AML patient. The mAb's used were identical as described under Figure 2a except that anti-CD33 was used instead of anti-CD19. Expression levels show that HLA locus A and B and total class I are higher on the tumor cells than on the patients normal cells.

Forward/side scatter dot plots of the different isolated cell populations are shown on the left.

Figure 3. Confirmations of complete HLA class I allelic losses on leukemic cells.

The patients malignant and normal cells were stained with the relevant human allele-specific anti HLA mAb's (black) and compared to irrelevant, isotypic control mAb's (white). The complete losses detected by CDC were all confirmed: A10 and A31 (case 1), B8 (case 7), A28 (case 29) and A1 (case 38). The weak reaction for B8 (case 38) scored clearly positive in CDC.

Table 1. Expression of locus A alleles on donor lymphocytes and cell lines.

HLA-A allele specific antibodies were tested against HLA class I typed donors and cell lines. Two isotype-matched controls were always included in every case tested. All antibodies reacted with all cells tested, except clone BR011F6. This antibody did not react with HLA A1104 (2 donors out of 4 tested). Reactivity is expressed as + (1 log), ++ (2 logs), +++ (3 logs) stronger than the negative controls used.

Table 2. Expression of locus B alleles on donor lymphocytes and cell lines.

HLA-B allele-specific antibodies were tested against HLA class I typed donors and cell lines. Conditions and interpretation of reactivity are identical as described under Table 1.

Table 3. Total HLA class I and locus-specific expression on lymphoid leukemic cells.

HLA expression on CLL-B and ALL-B at diagnosis was compared to the expression on normal peripheral blood B cells derived from healthy donors. All cells tested were untouched fractions derived from immunomagnetic isolation procedures. The results are expressed as mean fluorescence intensity values +/- SD. Comparison between patients and donor values was performed by means of the non-parametric Mann-Whitney U test.

Table 4. Total HLA class I and locus-specific expression on AML cells.

HLA expression on AML cells at diagnosis was compared to the expression on bone marrow derived CD33+ progenitors from healthy donors. All cells tested were untouched CD33+ cells obtained from immunomagnetic isolation procedures. Results and statistics as described in Table 3.

Table 5. Serological HLA-A and HLA-B typing on leukemic and normal cells.

Untouched leukemic and normal cells from patients at diagnosis were obtained using immunomagnetic procedures and tested for HLA class I expression by CDC.
(-: homozygous sample; ND: not done, not enough normal cells available; ■: no reaction with the respective anti-HLA antisera)

Table 6. Expression of locus A and B allospecificities on leukemic cells.

Allelic expression levels were tested by flow cytometry on malignant cells and compared to the patients remaining normal cells. All cells tested were untouched cell fractions obtained from immunomagnetic procedures. The results are expressed as mean fluorescence intensity (MFI) after background correction. The ratio R was derived from MFI malignant cells / MFI normal cells. An R value lower than 1 was considered as downregulation of that particular allele.
(H: homozygous; ND: not done, no specific antibody available)

Table 1: Expression of locus A alleles on donor lymphocytes and cell lines

Clone	mAb isotype	HLA class I Specificity	No donors tested	No cell lines tested	Reactivity in flow cytometry
VDK1D12	IgM, κ	A1	10	2	+++
SN66E3	IgM, κ	A2	22	3	++/+++
		A28	3	0	+++
OK2F3	IgM, κ	A3	11	2	+++
GV5D1	IgG1, λ	A1	10	2	+++
		A9	8	0	+++
NIE44B8	IgM, κ	A10	4	1	+/++
BR011F6	IgG1, λ	A11	4*	3	-/+++
HDG6B6	IgM, λ	A29,31,32,33 (19)	12	2	+
SN230G6	IgG1, λ	A2	22	3	+++
OK5A3	IgM, λ	A3	8	2	+++
		A11	3	3	+
		A1	10	2	+
OK4F9	IgM, κ	A1	8	2	+++
		A3	7	2	+++
		A11	3	3	+++
HDG2G7	IgG1, κ	A29,31,32,33 (19)	16	2	+++
OK1C9	IgM, λ	A3	11	2	+++
		A11	3	2	+
		A33 (19)	2	0	+
		A31 (19)	2	1	+

Table 2: Expression of locus B alleles on donor lymphocytes and cell lines

Clone	MAb isotype	HLA class I Specificity	No donors tested	No cell lines tested	Reactivity in flow cytometry
13E12	IgM, κ	B12	7	0	+++
GK31F12	IgM, κ	B13	4	0	+++
HA2C10B12	IgM, κ	B60(40)	7	2	+++
VTM3A1	IgG1, κ	B7	7	4	+++
VTM4D9	IgG1, κ	B7	7	4	++
DMS4G2	IgG1, λ	B62(15)	2	2	++/+++
		B35	7	2	+++
KAL3D5	IgG1, λ	B51(5)	5	2	+++
HDG8D9	IgG1, λ	B51(5)	5	2	+++
GR5B3	IgM, λ	B62 (15)	2	2	++
BVK5B10	IgM, κ	B8	7	1	+++
AE9D9	IgM, λ	B8	7	1	+
		B14	3	0	0
OK6H10	IgM, κ	B15	1	2	+++
		B35	7	2	+++
SN230G6	IgG1, λ	B17	3	0	+++
KG30A7	IgM, λ	B12	7	1	++
		B14	4	0	+++
GVK2F8	IgM, λ	B18	4	1	+++
		B39 (16)	3	0	+++
HDG2G7	IgG1, κ	B57 (17)	2	0	+++
OK6H10	IgM, κ	B35	7	2	+++
		B15	1	2	+++
		B15	1	2	+++
		B35	7	2	+++
FVS4G4	IgM, κ	B35	7	2	+++
		B17	6	0	+
		B62(15)	2	2	+++
		B51(5)	5	2	+++
		B14	5	0	+++
		B18	4	1	++/+++
		B38(16)	3	1	+++

Table 3: HLA expression on CLL-B and ALL-B

	Normal B-cells MFI (SD) (n=10)	CLL-B MFI (SD) (n=17)	p-value	ALL-B MFI (SD) (n=9)	p-value
Total class I	87 (33)	55 (39)	0.0067	32 (24)	0.0012
Locus A	28 (8)	22 (16)	NS	16 (8)	0.0002
Locus B	33 (14)	32 (14)	NS	16 (10)	0.005
β2m	69 (32)	52 (37)	NS	24 (15)	NS
Total class II	30 (7)	17 (13)	0.0134	22 (23)	NS
Locus DR	29 (2)	19 (15)	0.0452	26 (20)	NS

Table 4: ***HLA expression on bone marrow AML and CD33$^+$ normal donor progenitors***

	AML (n=20)	**CD33$^+$ normal progenitors (n=10)**	**p-value**
Total Class I	53 (32)	15 (11)	0.0032
Locus A	32 (22)	19 (10)	NS
Locus B	41 (26)	26 (15)	NS
β2 microglobulin	62 (52)	21 (19)	0.0119
Total Class II	28 (31)	7 (2)	0.0329
Locus DR	33 (31)	6 (2)	0.0013

Table 5: Results of the CDC serological HLA-A and HLA-B typing

Case No	Leukemic Subtype	Sample type	Normal Cells				Tumor Cells			
			A	A	B	B	A	A	B	B
1	CLL	BL	10	31	38	40	[redacted]		38	40
2	AML	BL	24	31	60	-	24	31	60	-
3	CLL	BL	2	3	7	51	2	3	7	51
4	CLL	BL	1	2	5	8	1	2	5	8
5	CLL	BL	3	10	35	44	3	10	35	44
6	AML	BL	24	32	39	57	24	32	39	57
7	CLL	BL	2	10	8	14	2	10	[redacted]	14
8	ALL	BL	2	3	60	-	2	3	60	-
9	CLL	BL	1	3	35	40	1	3	35	40
11	AML	BL	1	2	7	60	1	2	7	60
12	CLL	BL	3	23	7	44	3	23	7	44
13	CLL	BL	1	24	8	18	1	24	8	18
14	CLL	BL	24	32	35	51	24	32	35	51
15	AML	BL	ND	ND	ND	ND	3	11	35	38
16	CLL	BL	2	24	13	18	2	24	13	18
17	ALL	BL	1	-	5	7	1	-	5	7
18	ALL	BL	2	33	7	14	2	33	7	14
21	ALL	BL	ND	ND	ND	ND	10	18	52	-
23	AML	BL	2	32	8	17	2	32	8	17
26	CLL	BL	2	-	7	44	2	-	7	44
27	CLL	BL	2	-	8	44	2	-	8	44
28	CLL	BL	2	32	18	35	2	32	18	35
29	AML	BL	2	28	13	18	2	[redacted]	13	18
30	AML	BL	2	-	17	42	2	-	17	42
31	CLL	BL	11	33	35	57	11	33	35	57
32	AML	BL	2	3	39	51	2	3	39	51
33	AML	BL	2	23	35	44	2	23	35	44
34	AML	BM	1	2	13	60	1	2	13	60
38	CLL	BL	1	2	8	12	[redacted]	2	8	12
40	AML	BL	3	29	44	55	3	29	44	55
41	CLL	BL	3	29	51	60	3	29	51	60
42	CLL	BL	2	19	39	-	2	19	39	-
43	AML	BL	ND	ND	ND	ND	11	24	35	62
44	AML	BL	2	29	35	51	2	29	[redacted]	51
46	AML	BL	ND	ND	ND	ND	3	25	22	35
47	CLL	BL	1	3	7	8	1	3	7	8
48	AML	BL	ND	ND	ND	ND	1	2	8	60

Table 6: Expression of locus A and B allospecificities on leukemic cells

		HLA LOCUS A				HLA LOCUS B			
Case no	Leukemic subtype	HLA allele	Normal T cells	Malignant cells	R	HLA allele	Normal T cells	Malignant cells	R
3	CLL-B	A2	41.0	69.3	1.69	B7	63.8	57.7	**0.90**
		A3	92.5	184.9	1.99	B51	120.4	156.2	1.29
4	CLL-B	A1	20.7	19.8	**0.95**	B5	ND	ND	-
		A2	39.9	33.9	**0.84**	B8	59.3	57.7	**0.97**
13	CLL-B	A1	23.9	42.4	1.77	B8	83.1	43.7	**0.52**
		A24	29.2	38.6	1.32	B18	24.5	93.4	3.81
27	CLL-B	A2	30	18.2	**0.60**	B8	56.7	37.2	**0.65**
		H			-	B44	ND	ND	-
28	CLL-B	A2	29.2	31.9	1.09	B18	6.14	28	4.56
		A32	ND	ND	-	B35	57.2	36	**0.62**
29	AML	A2	21.1	14.1	**0.66**	B13	10.5	10.7	1.01
		A28	18.2	2.83	**0.15**	B18	2.14	1.97	**0.92**
30	AML	A2	16.8	10.9	**0.64**	B17	27.9	16.7	**0.59**
		H			-	B42	ND	ND	-
31	CLL-B	A1	25.7	29.0	1.12	B35	17.7	17.3	**0.97**
		A11	42.7	46.7	1.09	B57	38.6	39.1	1.01
32	AML	A2	29.1	29.6	1.01	B39	4.9	7.1	1.44
		A3	12.2	17.6	1.44	B51	34.7	47.3	1.36
33	AML	A2	7.6	13.9	1.82	B35	14.9	25.8	1.73
		H			-	H			-
34	AML	A1	57.3	78.1	1.36	B13	44.6	58.7	1.31
		A2	87.7	126.6	1.44	B60	12.1	20.1	1.66
43	AML	A3	6.46	7.33	1.13	B35	14.8	4.63	**0.31**
		A11	5.53	7.45	1.34	B62	15.4	6.85	**0.44**
45	AML	A1	19.2	5.98	**0.31**	B7	11.8	8.33	**0.70**
		A2	34.6	6.05	**0.17**	B60	9.46	8.18	**0.86**
50	ALL-B	A3	4.91	17.7	3.60	B7	12.1	23.0	1.9
		A26	5.07	17.9	3.53	B38	ND	ND	-
54	CLL-B	A1	20.1	6.79	**0.33**	B8	8.31	10.2	1.22
		A28	12.5	6.57	**0.52**	B49	ND	ND	-
55	CLL-B	A3	8.53	11.7	1.37	B7	19.3	10.8	**0.55**
		A33	ND	ND	-	B35	32.0	29.3	**0.91**
56	CLL-B	A2	32.5	4.75	**0.14**	B7	16.9	5.24	**0.30**
		A33	ND	ND	-	B60	12.9	10.5	**0.81**
57	AML	A3	7.05	7.89	1.11	B35	5.84	7.94	1.35
		A11	8.25	9.18	1.11	B39	ND	ND	-
61	CLL-B	A2	67.4	41.4	**0.61**	B13	70.9	64.0	**0.90**
		A3	15.9	7.8	**0.49**	B61	ND	ND	-
62	CLL-B	A32	6.1	10.5	1.72	B62	52.6	45.5	**0.86**
		H			-	H			-
63	CLL-B	A2	37.4	22.1	**0.59**	B44	7.4	7.1	**0.96**
		A31	3.7	3.1	**0.84**	B18	5.2	6.1	1.17
64	CLL-B	A2	50.3	37.3	**0.74**	B15	35.4	39.9	1.13
		A24	49.2	34.1	**0.69**	H			-
65	CLL-B	A32	37.2	61.0	1.64	B13	46.8	103.8	2.21
		A9	11	26.6	2.42	B39	2.8	13.3	4.75
67	CLL-B	A2	26.5	35.5	1.34	B8	50.5	212.5	4.21
		A24	20.6	137.5	6.67	B44	7.1	117.6	16.56
68	CLL-B	A28	45.1	40.8	**0.90**	B53	ND	ND	-
		A32	4.7	27.3	5.81	B7	23.5	43.1	1.83
69	CLL-B	A3	22.3	27.8	1.25	B35	17.1	28.6	1.67
		A10	4.2	5.9	1.40	B44	5.4	5.9	1.09
70	CLL-B	A26	90.1	64.4	**0.71**	B18	ND	ND	-
		H				B55	ND	ND	-
71	CLL-B	A1	31.9	43.9	1.38	B50	33.9	61.3	1.81
		A24	51.3	81.9	1.60	B62	59.9	87.9	1.47
72	CLL-B	A2	30.5	33.3	1.09	B35	34.4	49.6	1.44
		A3	22.8	25.0	1.10	B60	12.3	20.6	1.67
73	CLL-B	A11	43.1	25.2	**0.58**	B14	60.5	75.3	1.24
		A19	36.6	39.4	1.08	B51	30.2	36.5	1.21
74	CLL-B	A1	25.8	29.4	1.14	B8	55.9	57.0	1.02
		A2	36.4	34.2	**0.94**	B51	41.4	42.0	1.01
75	CLL-B	A2	20.3	29.6	1.46	B39	6.9	19.6	2.84
		A31	3.8	8.8	2.32	H			-